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Rifalazil Treats and Prevents Relapse of *Clostridium difficile*-Associated Diarrhea in Hamsters

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Although vancomycin and metronidazole effectively treat *Clostridium difficile*-associated diarrhea and colitis (CDAD), their use is associated with a high incidence of relapsing *C. difficile* infection. Rifalazil is a new benzoxazinorifamycin that possesses activity against *Mycobacterium tuberculosis* and gram-positive bacteria. Here we compared rifalazil and vancomycin for effectiveness in preventing or treating clindamycin-induced cecitis in a hamster model of CDAD. Golden Syrian hamsters were injected subcutaneously with clindamycin phosphate (10 mg/kg), followed 24 h later by *C. difficile* gavage. Hamsters received by gavage for 5 days vehicle, vancomycin (50 mg/kg), or rifalazil (20 mg/kg) either simultaneously with (prophylactic protocol) or 24 h after *C. difficile* administration (treatment protocol). While all vehicle-administered animals became moribund within 48 h of *C. difficile* administration, no rifalazil- or vancomycin-treated animals in either protocol showed signs of morbidity after 7 days. Ceca of rifalazil-treated animals showed absence of epithelial cell damage, significantly reduced congestion and edema, and less, but not statistically significantly less, neutrophil infiltration compared to those of vehicle-treated animals. In contrast, vancomycin-treated animals demonstrated severe epithelial cell damage and mildly reduced congestion and edema. Moreover, hamsters relapsed and tested *C. difficile* toxin positive (by enzyme-linked immunosorbent assay) 10 to 15 days after discontinuation of vancomycin treatment. None of the rifalazil-treated hamsters showed signs of disease or presence of toxins in their feces 30 days after discontinuation of treatment. Our results indicate that once daily rifalazil may be superior to vancomycin for curative treatment of CDAD.

Clostridium difficile is the most common cause of infectious nosocomial diarrhea (14), with reported incidence rates of 0.1 to 30 per 1,000 patients (2, 19, 29, 33). Prior treatment with antibiotics is an important precondition for *C. difficile* infection. Whereas some antibiotics, such as clindamycin, are more strongly associated with *C. difficile* infection, most antibiotics have some ability to dispose patients to infection (3). In susceptible individuals, disruption of the normal colonic microflora by antibiotic treatment leads to colonization by *C. difficile* (5). The bacterium then grows and releases two protein exotoxins, toxin A and toxin B, both of which mediate intestinal injury and inflammation in the human intestine (31, 34). Infected patients develop intestinal manifestations ranging from an asymptomatic state to mild diarrhea, severe pseudomembranous colitis, and toxic megacolon (17).

C. difficile-associated diarrhea (CDAD) is primarily treated by discontinuation of the precipitating antibiotic and administration of vancomycin or metronidazole (17). Both metronidazole and vancomycin are highly effective and associated with response rates of >96% (17). Metronidazole is currently considered the drug of choice for treatment of *C. difficile* infection because it is inexpensive and as effective as vancomycin (35). Side effects associated with metronidazole include nausea, vomiting, an unpleasant metallic taste, and peripheral neuropathy following prolonged therapy. Although highly successful as it relates to response rates and limited side effects, vancomycin is an alternative choice for treatment of CDAD. The main factors are its high cost and the spread of vancomycin-resistant enterococci in vancomycin-treated patients (13).

One of the main problems associated with treatment of CDAD is the high incidence (15 to 50%) of relapsing diarrhea following an initial successful response (8). Reinfection can be the result of persisting spores from the same strain or from a different *C. difficile* strain that can be acquired from the environment (36, 38). Relapsing CDAD represents a difficult and often challenging problem to the treating physician. A second course of the same antibiotic used to treat the initial episode is the most common initial therapy for recurrent *C. difficile*, with response rates of up to 92% (29). However, up to 65% of patients will develop further relapses (18). Treatment of this condition includes tapering the course or pulse dosing of vancomycin (24), use of probiotics such as *Saccharomyces boulardii* (26) or *Lactobacillus* sp. strain GG (10), combination of vancomycin and rifampin (7), use of anion-binding resins (16), and immunoglobulin therapy (21).

Rifalazil, also known as KRM-1648 or benzoxazinorifamycin, is a recently developed rifamycin derivative related to rifampin and rifabutin. Rifalazil possesses significant antimicrobial activity against a wide range of gram-positive and gram-negative bacteria, including mycobacteria (22), chlamydiae (32), and *Helicobacter pylori* (1). In vivo animal studies indicate that rifalazil possesses significant activity against tuberculosis-causing bacteria (11, 15). However, the activity of

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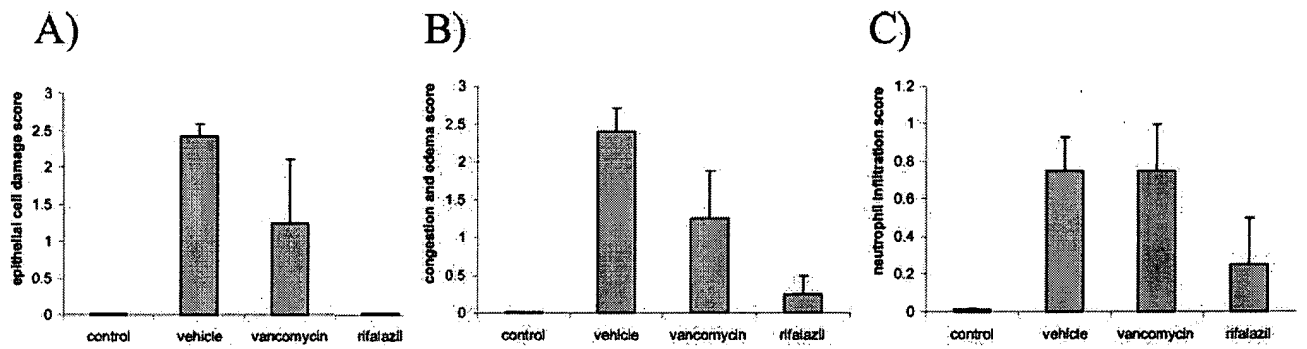

FIG. 1. Protective effect of rifalazil in *C. difficile*-associated histopathology in hamster cecum. A histologic evaluation of hematoxylin-and-eosin-stained cecal sections of hamsters exposed to *C. difficile* and various antibiotic treatments is shown.

this compound against *C. difficile* in vitro and in animal models of *C. difficile* infection in vivo has not been previously tested. In the present study, we tested the inhibitory activity of rifalazil against several *C. difficile* isolates and examined its therapeutic and prophylactic effects on clindamycin-induced cecitis in golden Syrian hamsters. Previous results indicate that this animal model closely resembles the *C. difficile*-induced diarrhea and colitis seen in humans (6), and it has been used to test the efficacy of several drugs currently used for treatment of *C. difficile* infection (4). The efficacy of rifalazil in preventing or treating clindamycin-induced cecitis was also compared to the effects of vancomycin in this animal model. Lastly, we compared the rates of relapse of CDAD in hamsters following discontinuation of rifalazil or vancomycin treatment.

MATERIALS AND METHODS

Materials. Rifalazil (ActivBiotics Inc., Cambridge, Mass.) was suspended in dimethyl sulfoxide (DMSO) at a concentration of 10 mg/ml with subsequent dilution in saline (NaCl, 0.9%) to reach a final concentration of 0.8% DMSO at the time of administration. Vancomycin (Sigma Chemical Co., St. Louis, Mo.) was dissolved in saline. Hamsters received either DMSO (0.8%), vancomycin (50 mg/kg/day) (27), or rifalazil (20 mg/kg/day) (20). Rifalazil was given orally once a day, while vancomycin was administered orally three times a day for 5 days.

MICs of rifalazil for *C. difficile* isolates. The MICs of rifalazil for 39 different *C. difficile* isolates were determined in accordance with the NCCLS guidelines (28). Isolates were subjected to dendrogram analysis (pulsed-field electrophoresis of SmaI-digested DNA from each isolate tested). This analysis was carried out at FOCUS Technologies (Herndon, Va.) in accordance with Fred Tenover's system for categorizing relatedness (37). The minority of strains that showed identical DNA banding patterns were determined to be clones rather than independent variants, and the redundant isolates were eliminated from the susceptibility analysis. Isolates were from the collection at FOCUS Technologies and were stored in 20% glycerol at −80°C.

Clindamycin-induced cecitis in hamsters. Golden Syrian hamsters (100 to 125 g) were housed in groups of two with free access to chow (Purina 5000) and tap water. Control animals received no treatment but were monitored for signs of disease. Three additional groups of animals received on day −1 a single subcutaneous injection of clindamycin (10 mg/kg), and after 24 h (day 0), hamsters were infected by gavage with 10^5 CFU of *C. difficile* strain 10463 (ATCC 43255). Hamsters were administered by gavage either vehicle, vancomycin, or rifalazil beginning at day 0. Half of the animals from each group ($n = 4$ per group) were sacrificed at day +6, and the remaining hamsters were observed for another 27 days (day +34). In another series of experiments, hamsters were treated with vehicle ($n = 10$), rifalazil ($n = 10$), or vancomycin ($n = 14$) for 5 days, with initiation of treatment 24 h after administration of *C. difficile*. Animals were observed for any sign of disease throughout the length of the experiment. At sacrifice (day +7), ceca were removed and washed in phosphate-buffered saline (PBS) and the contents were stored at −20°C for *C. difficile* toxin assays. Ceca were then fixed in formalin, paraffin embedded, stained with hematoxylin and eosin, and graded histopathologically by using parameters associated with *C. difficile*-associated mucosal damage and inflammation (30). Animal studies were approved by the institutional animal care and use committee.

Animal observations. Animals were observed three times a day for the duration of the experiment for mortality and morbidity and for the presence of diarrhea. Their weight was measured every other day. Animals judged to be in a moribund state were euthanized. Criteria used to assign a moribund state were extended periods (6 days) of weight loss, progression to an emaciated state, prolonged lethargy (more than 3 days), signs of paralysis, skin erosions or trauma, hunched posture, and a distended abdomen.

Detection of *C. difficile* toxins in cecal contents. At sacrifice, cecal contents were suspended in sterile PBS (pH = 7.4). Suspensions were clarified by centrifugation (20,000 × *g* for 5 min at 4°C), and the resulting supernatants were filtered through a 0.45-μm-pore-size filter and then stored at −20°C. The presence of *C. difficile* toxins (A and B) was detected with a commercially available enzyme-linked immunosorbent assay kit (*C. difficile* TOX A/B II; TechLab Inc., Blacksburg, Va.) in accordance with the manufacturer's instructions.

Microscopic damage evaluation. At sacrifice, ceca were removed, opened longitudinally, and washed in PBS. Full-thickness sections were fixed in formalin, paraffin embedded, and stained with hematoxylin and eosin, and the histologic severity of enteritis was graded by a "blinded" gastrointestinal pathologist (M.B.) by using parameters previously associated with toxin A-associated enterotoxicity (30).

Statistical analyses. Data were analyzed by Kaplan-Meier survival analysis, analysis of variance, or F test with the Statview statistical software program (Abacus Concepts, Berkeley, Calif.).

RESULTS

MIC_{90}s of rifalazil for *C. difficile* isolates. The MICs for 39 isolates of *C. difficile*, including two American Type Culture Collection strains and 37 clinical isolates, were determined. After dispensing with identical clones, determined by pulsed-field electrophoresis of DNA following SmaI digestion, the remaining strains were tested for susceptibility to rifalazil. Out of the MICs of rifalazil for the 31 isolates tested, only two were found to be greater than 0.5 μg/ml. The MIC for 50% of the strains tested (MIC_{50}) was 0.002 μg/ml, and the MIC for 90% of the strains tested (MIC_{90}) was 0.004 μg/ml. For *C. difficile* strain 10463, which was used in our in vivo studies, the MIC was 0.002 μg/ml.

Effect of rifalazil on *C. difficile*-associated histopathology. Histopathologic examination revealed that nontreated, *C. difficile*-infected hamsters demonstrated acute lesions in the cecal mucosa characterized by epithelial cell damage associated with heavy congestion and edema and significant neutrophil infiltration (Fig. 1). However, rifalazil-treated hamsters showed no epithelial damage ($P < 0.01$ versus vehicle-treated animals; Fig. 1A) and showed significantly reduced congestion and

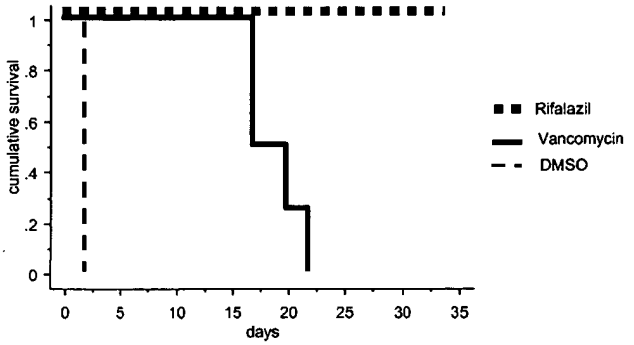

FIG. 2. Kaplan-Meier survival analysis of hamsters infected with *C. difficile* and treated at day 0 (prophylactic treatment) with vancomycin or rifalazil. All *C. difficile*-treated hamsters became moribund within 2 days after the challenge. All vancomycin-treated animals became moribund between days +14 and +24, while all rifalazil-treated animals survived for the duration of the observation period ($P < 0.05$, rifalazil versus vancomycin treatment, $n = 4$ per group).

edema of the mucosa ($P < 0.01$ compared to vehicle-treated animals; Fig. 1B). Furthermore, rifalazil-treated animals demonstrated a moderate reduction in neutrophil infiltration, but this result did not reach statistical significance (Fig. 1C). In contrast, vancomycin-treated hamsters were not protected from epithelial cell damage but showed reduced congestion and edema compared to vehicle-treated animals ($P < 0.05$; Fig. 1B).

Effect of rifalazil on *C. difficile*-associated mortality. Control animals that were not exposed to clindamycin and *C. difficile* showed no diarrhea and no sign of disease. Within 48 h of *C. difficile* administration, all vehicle (0.8% DMSO)-treated animals became moribund and were sacrificed (Fig. 2 and 3). In contrast, none of the animals treated for 5 days with rifalazil, either simultaneously (prophylactic protocol) or 24 h after *C. difficile* administration (therapeutic protocol), showed any sign of disease during treatment (Fig. 2 and 3). Similarly, vancomycin-administered animals on either the prophylactic or the treatment protocol demonstrated no disease symptoms during the course of vancomycin intake (Fig. 2 and 3).

Following discontinuation of vancomycin treatment, all of the animals in the prophylactic group and 65% of the hamsters in the treatment group became moribund within 14 to 24 days post-*C. difficile* challenge (Fig. 2 and 3). In sharp contrast, none of the rifalazil-treated animals in the prophylactic or treatment group showed any signs of disease up to 34 days post-*C. difficile* infection (Fig. 2 and 3). Moreover, *C. difficile* toxins A and B were detected by enzyme-linked immunosorbent assay in five of eight vancomycin-treated animals and none of the rifalazil-treated animals (zero of five; data not shown). These results indicate that, in contrast to discontinuation of vancomycin treatment, discontinuation of rifalazil treatment is not associated with relapsing CDAD.

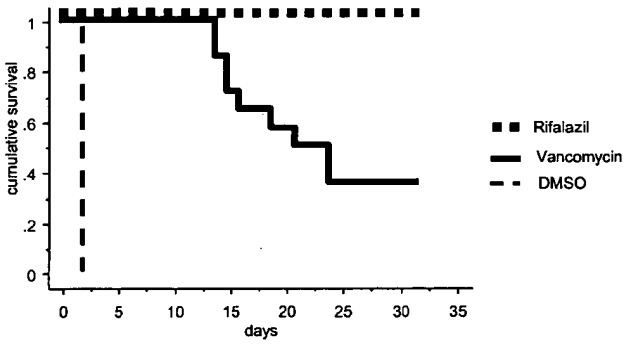

FIG. 3. Kaplan-Meier survival analysis of hamsters receiving treatment 24 h after a *C. difficile* challenge. All vehicle-treated animals became moribund within 2 days of the challenge. Only 35% of the vancomycin-treated animals survived after day +24 of the observation period, whereas 100% of the rifalazil-treated animals survived for up to 32 days of follow-up ($P < 0.001$, $n = 10$ to 14 per group).

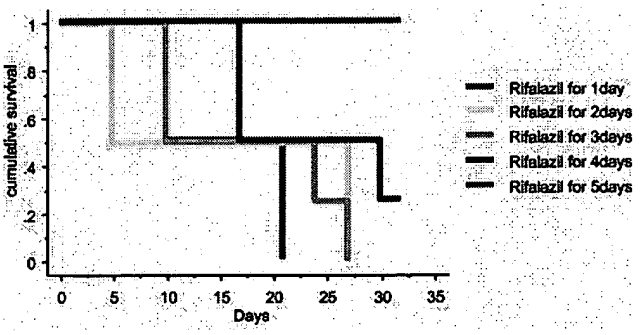

FIG. 4. Kaplan-Meier survival analysis of animals infected with *C. difficile* and receiving rifalazil treatment for 1 to 5 days. Four animals were treated for 1, 2, 3, 4, or 5 days. The analysis indicates that 5 days of rifalazil treatment is required for 100% survival of CDAD ($P < 0.01$ compared to other groups).

Effect of rifalazil treatment on *C. difficile*-associated weight loss. Clindamycin injection followed by oral administration of *C. difficile* resulted in significant weight loss compared to controls 2 days after *C. difficile* administration (10% of controls; $P < 0.05$, $n = 8$). At the end of rifalazil and vancomycin treatment, animals gained less weight (12 and 16% less, respectively; $P < 0.05$, $n = 8$ per group) compared to controls. Following discontinuation of vancomycin treatment, all of the animals in the prophylactic group started loosing weight 12 to 19 days post-*C. difficile* challenge (up to 50% loss compared to controls; $P < 0.05$). In contrast, following discontinuation of rifalazil, all of the animals in the prophylactic and therapeutic protocols started gaining weight in a time-dependent fashion similar to that of those in the control group. At the end of the observation period, the weight gain, expressed as a percentage of the day −1 body weight, of the animals receiving rifalazil treatment either on the day of the *C. difficile* challenge (day 0) or on the day after (day +1) was statistically indistinguishable from the weight gain of control animals ($P = 0.9$ by analysis of variance).

Time course effectiveness of rifalazil treatment. Having demonstrated that a 5-day course of rifalazil was effective against CDAD in hamsters, we evaluated the effectiveness of shorter rifalazil treatments. We administered rifalazil for 1, 2, 3, 4, and 5 days ($n = 4$ per group) 24 h after infection with *C. difficile* and monitored the animals for 32 days for signs of morbidity. The results (Fig. 4) demonstrate that 5 days of rifalazil treatment are required to obtain 100% survival of CDAD.

DISCUSSION

Our study demonstrates for the first time that rifalazil is highly active against many *C. difficile* strains in vitro and that 5 days of treatment with rifalazil prevents and treats clindamycin-induced, *C. difficile*-associated infection in Syrian hamsters in vivo. Moreover, rifalazil was more successful than vancomycin in several aspects of therapy for CDAD, including recurrence of *C. difficile* infection, histopathologic damage in the cecal mucosa, presence of *C. difficile* toxins in the cecal contents, and weight loss. These results strongly suggest that rifalazil could be used as a therapeutic or prophylactic agent in CDAD in humans.

Our results show a significant bactericidal effect of rifalazil against several *C. difficile* strains with an MIC_{50} of 0.002 μg/ml and an MIC_{90} of 0.004 μg/ml. Furthermore, no *C. difficile* toxins were detected in the ceca of rifalazil-treated animals, consistent with eradication of the microorganism. These results indicate a direct effect of rifalazil against this bacterial species that may account for the potent effects of this antibiotic in the hamster model of CDAD observed in our study. Although the mechanism of this response was not directly examined here, it is likely that rifalazil exerts its bactericidal effect against *C. difficile* by inhibiting bacterial RNA polymerase as previously described in studies with RNA polymerase derived from *Mycobacterium avium* and *Escherichia coli* strains (9). Moreover, the potent anti-*C. difficile* activity exerted by rifalazil in our in vitro and in vivo experiments is consistent with its antimicrobial effects against several other enteric bacteria (32).

CDAD is an increasing problem in hospitals primarily because of the wider use of broad-spectrum antibiotics (17). Antibiotic therapy with metronidazole or vancomycin is the most common form of treatment of this infection, but antibiotic use is associated with frequent disease recurrence (18, 25). Thus, it is generally believed that an ideal agent for the treatment of this disease would be effective in controlling the in vivo growth of *C. difficile* and the ensuing pathogenic consequences. This agent should also protect against relapse and not predispose the recipient to CDAD. On the basis of this principle, we also evaluated whether rifalazil has an effect in relapsing CDAD in the hamster model and compared it to the antimicrobial vancomycin. The results presented here indicate that, in contrast to vancomycin, rifalazil administration is not associated with relapsing CDAD. It is noteworthy that of 18 animals treated with rifalazil and monitored for 34 days, none relapsed. Although specific experiments to measure concentrations of rifalazil in the feces or tissues of treated hamsters were not performed in our study, prior pharmacokinetic studies allow us to speculate on the possible mechanisms of this "relapsing-preventive" response. It is well established that compared to other members of the rifamycin group of drugs, rifalazil has a long half-life, significant intracellular concentrations, and a large volume of distribution (reviewed in reference 32). For example, following oral administration of single rifalazil doses to rats and dogs, it was demonstrated that the terminal half-life ranged from 6.2 to 19.5 h in the former and from 15.2 to 24 h in the latter (12). Moreover, rifalazil concentrations in the tissue of rats were 277 times higher than those in their blood, consistent with its large volume of distribution (~10 liters/kg) (12). A long half-life for rifalazil was also demonstrated in humans (23). Thus, the possibility of a very high tissue intracellular drug concentration, coupled with its long half-life, may account for the potent effects of rifalazil on *C. difficile* infection seen in our study.

In summary, our results indicate that rifalazil is highly successful when administered prophylactically, as well as therapeutically, in CDAD in hamsters. Our finding that rifalazil, in contrast to vancomycin, is not associated with reappearance of *C. difficile* infection following its discontinuation in this animal model indicates that it can be superior to vancomycin (and possibly to metronidazole, whose use is also associated with relapsing *C. difficile* infection) as a first line of treatment for CDAD. This is the first demonstration of prophylactic, as well as therapeutic, use of rifalazil in *C. difficile* infection.

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